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Leukemia inhibitory factor blocks early differentiation of skeletal muscle cells by activating ERK

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Abstract

Leukemia inhibitory factor (LIF) is a multifunctional cytokine belonging to the interleukin-6 family and has been shown to stimulate regeneration of injured skeletal muscle. Although LIF has been shown to stimulate muscle cell proliferation, its precise role in differentiation is unclear. Thus, we examined the effect of LIF on the differentiation of cultured C2C12 myoblast cells. In this study, we used both non-glycosylated LIF expressed in bacteria and glycosylated LIF secreted from NIH3T3 cells infected with Ad-LIF. Both non-glycosylated and glycosylated LIF blocked differentiation of myoblasts as measured by expression of myosin heavy chain and myotube formation. Treatment of myoblasts with LIF induced phosphorylation of ERK, and the LIF-induced inhibitory effect on myogenesis was blocked by pretreatment with U0126, a specific MEK inhibitor, and transient transfection with dominant negative (DN)-MEK1. In contrast, although LIF activated STAT3, the LIF-induced repression of the MCK transcriptional activity was not reversed by pretreatment with AG490, a specific Jak kinase inhibitor or transient transfection with DN-STAT3. Additionally, LIF exhibited its inhibitory effect on myogenesis only when cells were treated at earlier than 12 h after inducing differentiation. Taken together, these results suggest that LIF strongly inhibited early myogenic differentiation though activation of the ERK signaling pathway and its effect is irrespective of glycosylation.

Keywords: LIF; Muscle; Differentiation; Adenovirus; ERK; Regeneration

1. Introduction

Leukemia inhibitory factor (LIF) is a pleiotrophic glycoprotein that belongs to the interleukin-6 (IL-6) cytokine family, which includes ciliary neurotrophic factor, oncostatin M and cardiotrophin-1. It is secreted by various cell types and mediates a variety of biological effects depending on the target tissue and cell lineage. The intracellular signaling pathways responsible for LIF action involve the activation of Jak/Tykotyrosine kinases and STAT transcription factors [1]. Following ligand-induced dimerization of receptors, gp130 and LIF receptor β (LIFRβ), gp130 is phosphorylated by Jak/Tyk kinases, which are constitutively associated with it. The activated

Abbreviations: Ad, adenovirus; LIF, leukemia inhibitory factor; LIFRβ, leukemia inhibitory factor receptor β; IL-6, Interleukin 6; bFGF, basic fibroblast growth factor; TGF-β, transforming growth factor-β; IGF-I, insulin like growth factor-I; Man-6-P, mannose 6-phosphate; MHC, myosin heavy chain; MCK, muscle creatine kinase; Luc, luciferase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK1, mitogen-activated protein kinase-1; STAT, signal transducers and activators of transcription; DN-MEK1, dominant-negative MEK1; DN-STAT3, dominant-negative STAT3; PIAS3, protein inhibitor of activated STAT3

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Jak/Tyk then phosphorylates either STAT1, STAT3 or STAT5, which subsequently translocates to the nucleus, where it transactivates LIF-responsive genes. In addition, a different part of the gp130 subunit serves as a docking site for SHP2, which recruits Grb2 and leads to the activation of Ras/mitogen-activated protein kinase (MAPK) [2].

In skeletal muscle, LIF has been shown to stimulate muscle regeneration. It is usually expressed at a low level in normal muscle [3,4], but is up-regulated in denervated or injured muscle [4–8] and in the muscle of *mdx* dystrophic mouse [9]. In addition, mRNAs of gp130 and LIFRβ are significantly up-regulated following muscle crush injury in the regenerating muscle precursor cells, but are not detected in newly formed myotubes [10]. Thus, expression of LIF signaling molecules seems to be associated with the early timing of muscle regeneration. Administration of LIF to *mdx* mouse muscle following myoblast transplantation or to the injured LIF null mutant mice also enhanced regeneration [11,12]. These myotrophic actions of LIF suggest that it behaves as a trauma factor for injured muscle. In contrast, however, periodic and systemic administration of LIF to bupivacaine-induced muscle degeneration mice did not stimulate regeneration [13], and no significant effect of LIF on myotube formation in injured BALB/c mouse [14] was found. Thus, these contradictory observations lead to ambiguity in the actual role of LIF in muscle regeneration.

Although LIF has been shown to stimulate the proliferation of myoblast in vitro [15–17], its role in differentiation is unclear. A previous report showed that LIF had no significant effect on myogenesis of muscle satellite cells, but it was not until 5 days after treatment with LIF that differentiation was induced by switching the growth medium to differentiation medium [18]. In the present study, we therefore re-examined its effect on myogenic differentiation by treating C2C12 cells with LIF at the initial phase of differentiation. We used both non-glycosylated and glycosylated LIF in order to examine any differences in its action on differentiation. Our results demonstrated that both forms of LIF strongly inhibited early myogenic differentiation and that this effect was mediated though ERK phosphorylation.

2. Materials and methods

2.1. Materials

NIH3T3 cells, 293 cells, M1 cells (TIB 192) and C2C12 myoblast cells (CRL 1772) were purchased from ATCC (Manassas, VA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), TRIZOL reagent and other culture materials were obtained from Gibco BRL (Gaithersburg, MD). Adenovirus expression vector kit and T4 DNA polymerase were purchased from TakaRa (Japan). LIF was purchased from Chemicon (Temecula, CA), and U0126 and AG490 from Biomol (Plymouth Meeting, PA). The anti-ERK, phospho-ERK, $p21^{waf1/cip1}$ and α-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-STAT3 and phospho-STAT3 antibodies from Cell Signaling Technology (Beverly, MA). Anti-myogenin (F5D) and LIF antibodies were obtained from Dako (Denmark) and R&D Systems (Minneapolis, MN), respectively. Chemicals including PMSF, aprotinin and leupeptin, and anti-Flag (M2) antibody were purchased from Sigma (St. Louis, MO).

2.2. Cell cultures

NIH3T3 cells, 293 cells and C2C12 myoblast cells were grown in the growth medium (GM), DMEM supplemented with 10% FBS, penicillin (50 U/ml), and streptomycin (100 μg/ml), and M1 cells in RPMI 1640 including 10% FBS, penicillin (50 U/ml) and streptomycin (100 μg/ml). Cells were maintained at 37 °C in humidified 95% air and 5% CO_2 atmosphere.

2.3. Myotube counting

To induce muscle differentiation, C2C12 cells were seeded in 24-well plates at 1×10^4 cells/well in the GM, allowed to grow to approximately 60%–80% confluence, and then switched to differentiation medium (DM; DMEM with 0.5% calf serum). Three days later, the number of multinucleated myotubes present in a field (1 $\mu m^2\times100$) was counted under a phase-contrast microscope (Olympus PM 20).

2.4. Plasmids, DNA transfections, and luciferase assays

The expression vector for dominant negative (DN)-MEK1 (S218A, S222A) [19] was a generous gift from Dr. K.Y. Choi (Yonsei University, Korea). The expression vector for DN-STAT3 (Y705F) and the luciferase reporter plasmid with three repeated STAT sites (pZLuc-TK) were kindly provided by Dr. J.E. Darnell, Jr. (Rockefeller University). The luciferase reporter plasmid with promoter region of muscle creatine kinase (MCK-Luc) [20] and the expression vector for mouse PIAS3 were kind gifts of Dr. K.Y. Lee (Chungbuk National University, Korea) and Dr. K. Shuai (University of California), respectively. C2C12 cells were transfected by using Lipofectamine (Invitrogen, De Schelp, Netherlands). Briefly, 4×10^4 cells per well were plated onto 12-well plates. On the following day, cells were transfected with various combinations of plasmids as indicated in the figure legends. The total amount of DNA used for each well was normalized with the relevant empty expression vehicle. At 24 h after transfection, cells were treated with LIF (20 ng/ml) in DM for the indicated times or kept in DM for 48 h. Cells were then harvested and assayed for luciferase activity using the Luciferase assay system (Promega, Madison, WI) in the Lumin meter LB 9505 (Berthold, Germany). The transfection efficiency was normalized for β-galactosidase activity.

2.5. Western blot analysis

To examine expression levels of myosin heavy chain (MHC), $p21^{waf1/cip1}$ and myogenin, and activation of ERK and STAT, C2C12 cells were treated with LIF (20 ng/ml) in DM for the indicated times, washed once with PBS and lysed with modified RIPA buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EGTA, 0.1% SDS, 1 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 1 μg/ml aprotinin and 1 μg/ml leupeptin). The lysate was centrifuged at 13,000×*g* for 10 min at 4 °C and the supernatant used for the experiment. An aliquot (50 μg) of protein was separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and electrotransferred onto 0.2-μm nitrocellulose membrane using Towbin transfer buffer (192 mM glycine, 25 mM Tris, 20% methanol, pH 8.3). The membrane was preincubated with PBS containing 5% skim milk, and probed with monoclonal anti-MHC (MF20), polyclonal anti-$p21^{waf1/cip1}$ (1:1000), monoclonal anti-myogenin (F5D, 1:500), polyclonal anti-actin (1:1000), polyclonal anti-ERK (1:1000), monoclonal anti-phospho ERK (1:1000), polyclonal anti-STAT3 (1:1000), polyclonal anti-phospho STAT3 (1:1000), monoclonal anti-Flag (1:1000) or polyclonal anti-LIF (1:1000) in PBS containing 5% skim milk for 1 h at room temperature. The membrane was then washed with PBS containing 0.03% Tween 20 and incubated with an appropriate horseradish peroxidase (HP)-conjugated secondary antibody. After several washes, the blot was developed using an enhanced chemiluminescence reagent (ECL, Amersham Biosciences, Uppsala, Sweden) according to the manufacturer's instructions.

Culture media from NIH3T3 cells infected with Ad-LIF were retrieved at the indicated times and centrifuged at 13,000×*g* for 10 min at 4 °C. Supernatants were concentrated fivefold using Ultrafree®-4 centrifugal filter (Millipore, Bedford, MA) and the amount of LIF was assayed by Western blot analysis as described above. The protein concentration was determined by the BCA method (Sigma).

2.6. Construction of a recombinant adenovirus

In order to construct a recombinant adenovirus encoding LIF, cDNA of rat LIF was cloned into the pGEM-T easy vector (Promega) by reverse transcriptase-polymerase chain reaction (RT-PCR) using total RNA prepared from rat retina. Its cDNA sequence was confirmed using an ABI3700 Automatic Sequencer (PE Biosystems, Foster City, CA). After full-length LIF cDNA was blunt-ended with T4 DNA polymerase, it was inserted into the *Swa*I site of the cosmid pAxCAwt (designated pAxCAwtLIF; Fig. 5A). Thus, the inserted cDNA was transcribed under the control of a CAG promoter, which is composed of cytomegalovirus enhancer, chicken β-actin promoter and a part of the 3′ untranslated region of rabbit β-globin. The recombinant virus was produced according to the protocol provided by the supplier. Briefly, the recombined cosmid DNA was co-transfected with the *Eco*T221 digested DNA-terminal protein complex into 293 cells to generate the recombinant virus by homologous recombination. The recombinant virus, designated as Ad-LIF, was then propagated in 293 cells. After the third propagation, virions were released by breaking 293 cells with repeated freezing-thawing. The cell lysate was centrifuged at 1000×*g* for 10 min at 4 °C to remove the cell debris, and the supernatant stored at −80 °C. The titre of recombinant virus was determined by the modified end-point cytopathic effect assay on 293 cells [21] and expressed in plaque-forming units (pfu). The Ad-LacZ virus was prepared using the pAxCAiLacZ cosmid provided by the supplier and used as a control.

2.7. Preparation of recombinant glycosylated LIF

In order to obtain recombinant glycosylated LIF, NIH3T3 cells were infected with Ad-LIF at a multiplicity of infection (moi) of 5 according to the procedures described elsewhere [21]. No cytotoxic effect on viability and morphology was observed following virus infection. The culture medium harvested on day 5 after infection was centrifuged at 13,000×*g* for 10 min at 4 °C, and the supernatant containing LIF was used for the experiments. The expression level and biological activity of LIF were determined by Western blot analysis using an anti-LIF antibody and by the phagocytic ability of M1 cells, respectively.

2.8. Measurement of phagocytic activity of LIF

M1 cells were incubated with polystyrene latex beads (2 μl/ml, average diameter 0.88 μm; Sigma) in the presence of the NIH3T3 cell culture medium (1.5 μl/ml) infected with Ad-LacZ or Ad-LIF for 12 h at 37 °C in a 5% CO_2 incubator. The cells were then washed three times with Ca^{2+}- and Mg^{2+}-free PBS and photographed under a phase-contrast microscope.

2.9. RT-PCR

Total RNA from NIH3T3 cells infected with or without Ad-LIF was prepared using TRIZOL reagent. The first strand cDNA was synthesized by the RT reaction. Briefly, 2 μg of total RNA was incubated in a tube containing 10 U of AMV reverse transcriptase (Promega), 0.5 mM dNTPs and 0.5 μM oligo$(dT)_{17}$ for 1 h at 42 °C. Then, PCR was performed in a total volume of 50 μl containing 1 μl of cDNA, 50 mM KCl, 10 mM Tris–HCl (pH 9.0), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 20 pmol of a primer pair (sense: 5′-CTACTGCTCATTCTGCACTG-3′, antisense: 5′-CCAAC-TTCTTCCTTTGGAAG-3′) specific to rat LIF and 2.5 U of *Taq* DNA polymerase (Promega). Samples were subjected to 30 cycles of PCR according to the following scheme: at 94 °C for 30 s, at 58 °C for 30 s and at 72 °C for 1 min using

the GeneAmp® PCR Thermocycler System 2400 (PE Biosystems). PCR products were electrophoresed on a 1.2% agarose gel and visualized by staining with ethidium bromide.

2.10. Immunocytochemical staining

C2C12 cells treated with or without LIF in DM for 3 days were fixed with 4% paraformaldehyde prepared in PBS for 30 min at room temperature. The cells were washed three times with PBS and incubated in 100% methanol containing 0.1% hydrogen peroxide for 10 min at −20 °C. The myotubes were incubated with PBS containing 10% horse serum, 0.2% Triton X-100 and 2% BSA for 30 min at room temperature, washed with PBS, and then incubated with monoclonal anti-MHC antibody (MF20) prepared in PBS containing 2% horse serum for 2 h at room temperature. After a thorough rinse with PBS, the myotubes were incubated with biotin conjugated anti-mouse IgG (Santa Cruz, 1:500) for 2 h at room temperature. Immunoreactivities were visualized by using a DAB staining kit (Vector, Burlingame, CA).

3. Results

3.1. LIF inhibits myogenic differentiation

To examine the effect of LIF on muscle differentiation, the C2C12 myoblast cells grown in the GM containing 10% FBS were switched to DM containing 0.5% calf serum to induce differentiation, and treated with LIF for 3 days. Differentiation of myoblasts was assayed by counting the number of myotubes in a field ($1\mu m^2 \times 100$) under a light microscope. Myotubes having two to four nuclei were observed in the untreated dishes while no myotube was seen in the dishes treated with LIF (Fig. 1A). The number of myotubes in the LIF treated dishes decreased in a dose-dependent manner (Fig. 1B); ED_{50} was approximately 1 ng/ml. To further characterize the inhibitory effect of LIF on differentiation, we examined the expression level of differentiation marker proteins such as MHC, $p21^{waf1/cip1}$ and myogenin in cells treated with or without LIF. As shown in Fig. 1C, compared with differentiating cells, the level of MHC in LIF-treated cells was almost completely blocked, and that of $p21^{waf1/cip1}$ was also reduced. The expression of myogenin, although to a less extent, was delayed compared with control cells. These results suggest that LIF strongly inhibits muscle differentiation.

3.2. Phosphorylation of ERK is responsible for the LIF-induced inhibition of myogenesis

To identify signaling pathways involved in LIF-induced inhibition of myogenesis, we examined the phosphorylation of ERK and STAT proteins following the treatment of C2C12 myoblasts with LIF (20 ng/ml) in DM. As previously reported [22], treatment of C2C12 cells with LIF caused rapid phosphorylation of ERK and STAT3 (Figs. 2A and 3A), but not STAT1 (data not shown); the

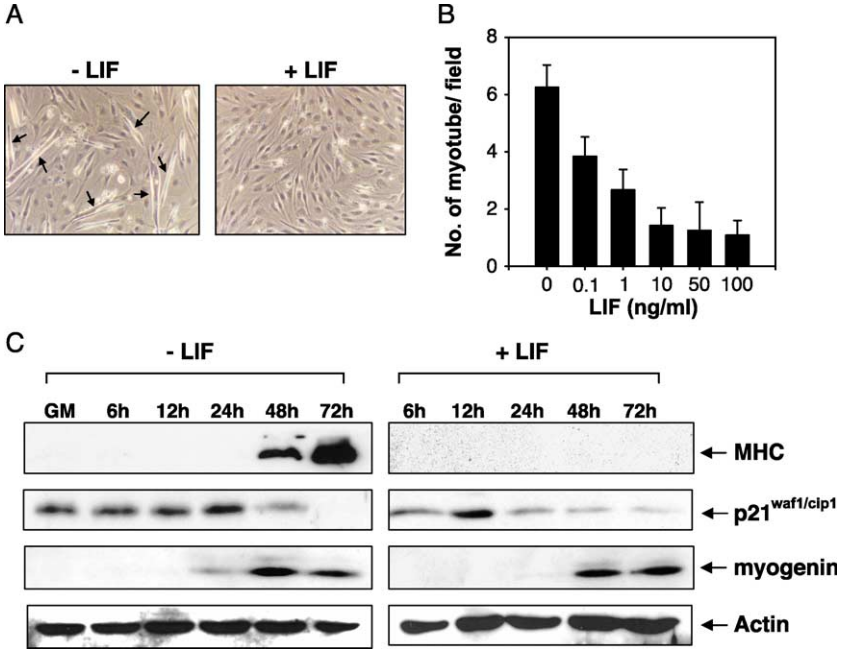

Fig. 1. LIF inhibits muscle differentiation. (A) C2C12 cells grown in the GM were switched to DM in order to induce muscle differentiation and treated with LIF (20 ng/ml) for 3 days. Phase-contrast images (a, b) of the cells treated with or without LIF are shown. Arrows show myotubes. (B) shows the number of myotubes per field (1 μm^2, ×100) in the cultures treated at various concentrations of LIF. Data are means±S.D. of triplicate experiments. (C) The expression levels of MHC, myogenin and $p21^{waf1/cip1}$ proteins in the cells treated with or without LIF for the indicated times were examined by Western blot analysis.

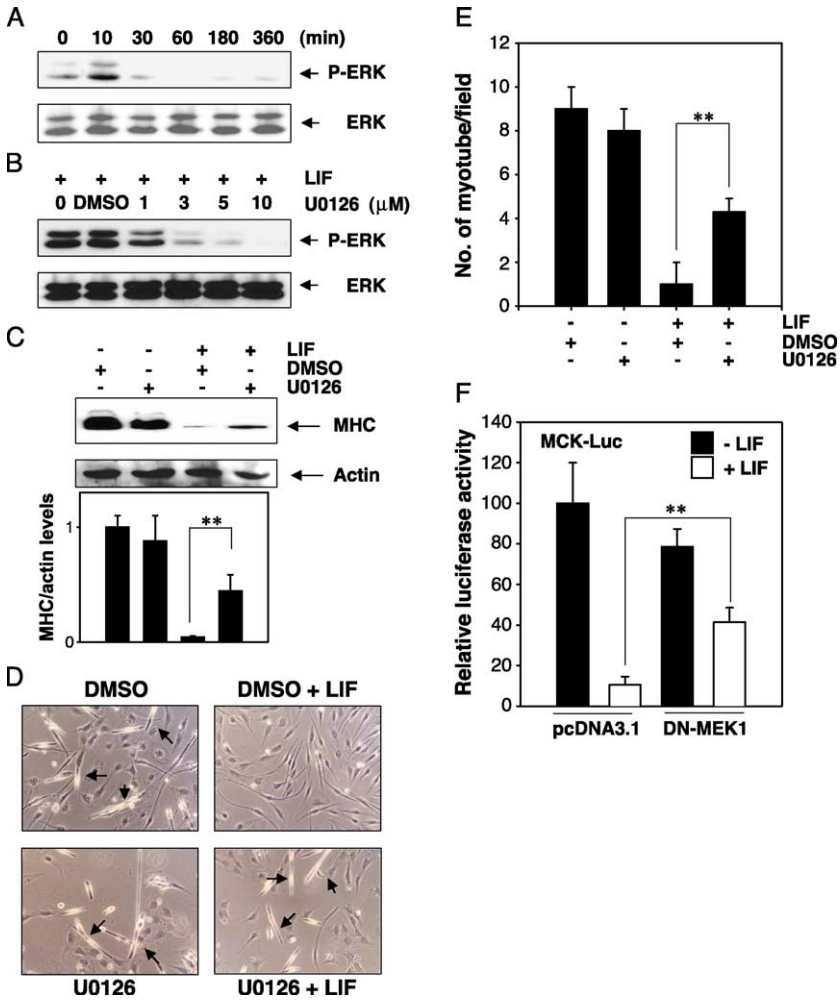

Fig. 2. Activation of the ERK pathway by LIF leads to the inhibition of myogenesis. (A) C2C12 cells grown in the GM were switched to DM and treated with LIF (20 ng/ml) for 0–360 min. Total protein fraction was prepared as described in Materials and methods, and phosphorylation of ERK was examined by Western blot analysis using anti-phosphospecific ERK antibody. (B) Cells grown in the GM were switched to DM and pretreated with various concentration of U0126, a MEK inhibitor, for 30 min followed by LIF treatment for 10 min. After then, ERK phosphorylation was examined by Western blot analysis using an anti-phosphospecific ERK antibody. The concentration of U0126 at which ERK phosphorylation was blocked was approximately 3 μM. (C, E) Cells in the DM were pretreated with U0126 (3 μM) for 30 min and then treated with LIF (20 ng/ml) for 3 days. The differentiation of the cells was determined by examining the expression of MHC protein by Western blot analysis with anti-MHC antibody (MF20) (C) or by counting the number of myotubes per field (E, 1 μm^2, ×100). (C) shows a representative Western blot and its relative quantitation from three independent experiments. (D) shows phase-contrast images of the cells (×100) and arrows indicate myotubes. (F) Cells were transfected with 500 ng of MCK-Luc together with either 200 ng of pcDNA3.1 (Invitrogen) or DN-MEK1 by using Lipofectamine. pcDNA3.1/*lacZ* (100 ng) was co-transfected to normalize the transfection efficiency. At 24 h after transfection, cells were treated with LIF (20 ng/ml) for 48 h following switching the GM to DM. Cells were then harvested and assayed for luciferase activity. The results are shown as the mean±S.D. from three independent experiments. (**P<0.01).

ERK and STAT3 activation were maximal at 10 min and decreased almost to control levels at 30 and 60 min, respectively.

Activation of the ERK pathway seems to be important for mediating the repressive effect of growth factors on myogenesis [23–27] although another report has suggested that the ERK activation positively regulates myogenesis [28]. Therefore, to test if ERK activation by LIF mediates the inhibition of myogenesis, we used U0126, a specific MEK inhibitor. When the cells were pretreated with U0126 (1–10 μM) for 30 min, ERK phosphorylation was reduced in a dose-dependent manner (Fig. 2B). In addition, pretreatment with U0126 (3 μM, 30 min) partially reversed the LIF-induced (20 ng/ml, for 3 days) reduction of MHC protein expression (Fig. 2C) and blocked LIF-induced inhibition of muscle differentiation (Fig. 2D and E).

Both the transcription and enzyme activity of muscle creatine kinase (MCK) are known to increase as myoblast cells differentiate. Thus, we examined the transcriptional level of MCK by assaying the luciferase activity in MCK-Luc transfected cells in the presence or absence of LIF. As expected, transcriptional activity of the MCK gene was dramatically decreased in LIF-treated cells (Fig. 2F). Interestingly, the LIF-induced repressive effect on MCK promoter activity was significantly reversed in cells

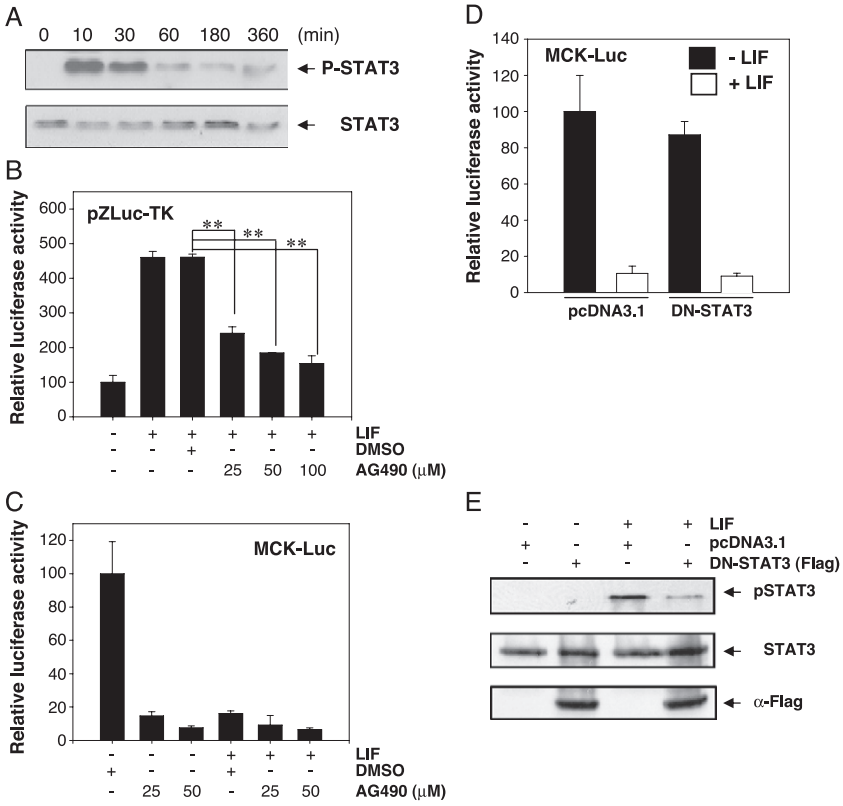

Fig. 3. Activation of STAT3 by LIF is not involved in the inhibition of myogenesis. (A) Phosphorylation of STAT3 in C2C12 cells treated with LIF (20 ng/ml) for 0–360 min was detected by Western blot analysis using anti-phosphospecific STAT3 antibody. (B, C) Cells were transfected with either 500 ng of pZLuc-TK (B) or 500 ng of MCK-Luc (C) together with 100 ng of pcDNA3.1/*lacZ* by using Lipofectamine. At 24 h after transfection, cells were switched to DM, pretreated for 30 min with various concentration of AG490, a Jak kinase inhibitor, and treated with LIF for 6 h (B) or 48 h (C). Cells were then harvested and assayed for luciferase activity. (D) Cells were transfected with 500 ng of MCK-Luc together with either 200 ng of pcDNA3.1 or DN-STAT3 by using Lipofectamine. pcDNA3.1/*lacZ* (100 ng) was cotransfected to normalize the transfection efficiency. At 24 h after transfection, cells were switched to DM and treated with LIF (20 ng/ml) for 48 h. Cells were then harvested and assayed for luciferase activity. The results are shown as the mean±S.D. of triplicate experiments. (E) Cells were transfected with 1 μg of pcDNA3.1 or 1 μg of DN-STAT3 by using Lipofectamine. The media was changed to DM on the following day and the cells were treated with LIF (20 ng/ml) for 10 min and analysed for phosphorylation of STAT3 by Western blot analysis. (**P<0.01).

transiently transfected with DN-MEK1 (Fig. 2F), suggesting that LIF-induced muscle differentiation is blocked by the ERK signaling pathway.

Recently, it was reported that activated STAT3 also inhibits myogenic differentiation though direct interaction with MyoD [29]. Thus, to examine whether STAT3 activation by treatment of LIF is also involved in the inhibitory effect on myogenesis, we used AG490, a specific Jak kinase inhibitor [30,31]. First, we tested whether AG490 is able to inhibit STAT3 activation induced by LIF using the luciferase reporter plasmid containing three repeated STAT binding sites (pZLuc-TK). As expected, the LIF-induced STAT3 transcriptional activity was blocked by pretreatment with AG490 in a dose-dependent manner in cells transiently transfected with pZLuc-TK (Fig. 3B). Next, we examined whether AG490 inhibits LIF-induced muscle differentiation by measuring promoter activity of the MCK gene. In contrast to blocking of ERK, the repression of MCK transcriptional activity induced by LIF was not reversed by AG490. Similar results were observed in cells transiently transfected with DN-STAT3 (Fig. 3D) or PIAS3, a STAT3 inhibitor (data not shown). Furthermore, the pretreatment of cells with AG490 alone caused dramatic reduction in the basal MCK activity (Fig. 3C). Transfection of DN-STAT3 and functional blocking of STAT 3 phosphorylation were confirmed (Fig. 3E). These results clearly demonstrate that LIF induces inhibition of myogenesis through ERK activation, but not STAT3.

3.3. Inhibition of myogenesis by LIF requires critical timing of treatment

It has been reported that there is a critical time frame (16–20 h) after plating during which bFGF induced ERK activity is able to repress myogenic gene expression and differentiation [25]. Thus, we tested whether LIF causes myogenic repression only during a restricted period after initiation of differentiation. To do this, we analyzed the level of MHC expression and MCK promoter activity after treating cells with LIF at various time points before and after switching the GM to DM. As shown in Fig. 4, LIF treatment of cells between 0 and 12 h after switching to DM

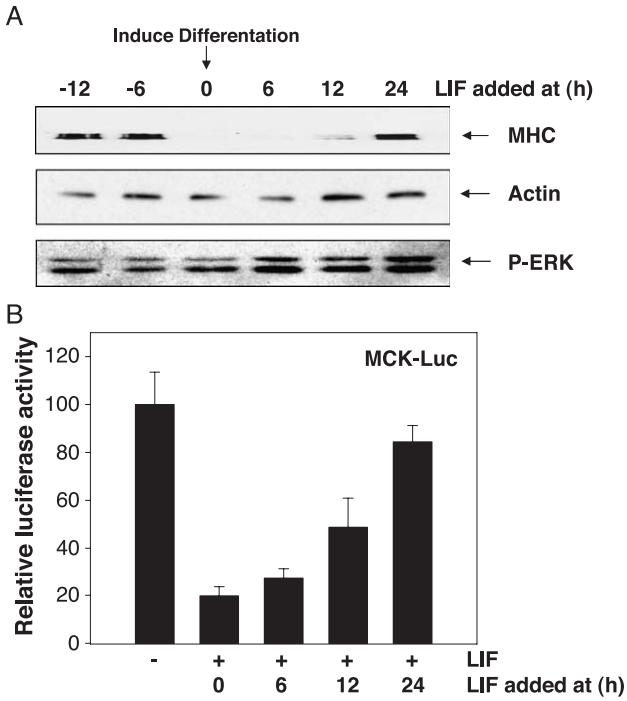

Fig. 4. LIF-induced inhibition of myogenesis requires critical timing of treatment. (A) C2C12 cells in the GM were switched to DM, treated with LIF (20 ng/ml) at the indicated times before or after the media change, and incubated for 3 days (MHC and actin) or 10 min (pERK). Total protein fraction was prepared as described in Materials and methods. MHC protein level was examined by Western blot analysis using anti-MHC (MF20) antibody and ERK phosphorylation using anti-phosphospecific ERK antibody. (B) Cells were transfected with 500 ng of MCK-Luc and 100 ng of pcDNA3.1/*lacZ* by using Lipofectamine in the GM. Cells were switched to DM on the following day, treated with LIF at the indicated times before or after the media change, and further incubated for 48 h. MCK promoter activity was measured by the luciferase assay. The results are shown as the mean±S.D. of triplicate experiments. Zero time indicates when the GM was switched to DM.

effectively inhibited both MHC expression (Fig. 4A) and MCK transcriptional activity (Fig. 4B) while the addition of LIF to proliferating myoblasts in the GM or to differentiating myoblasts which had been incubated for 24 h in the DM did not. However, ERK was activated in cells treated at any time points of the experimental scheme, indicating that the ability of LIF to activate ERK is not lost through the time points and therefore is not the reason for the ineffectiveness of LIF at these time points. These results suggest that LIF exhibits its action on the early phase of muscle differentiation, and ERK activation is sufficient only during a limited time window.

3.4. Glycosylated LIF secreted from Ad-LIF-infected NIH3T3 cells is biologically active

Ad-LIF was constructed according to the protocol provided by the supplier (TakaRa) and infected into NIH3T3 cells as described in Materials and methods. Transcription of the LIF gene under the control of CAG promoter was measured by RT-PCR using a pair of specific primer complementary to rat LIF cDNA. As shown in Fig. 5B, LIF mRNA was expressed in the cells infected with the recombinant adenovirus but was absent in the cells without infection. This result indicated that the LIF gene was efficiently transcribed in NIH3T3 cells, and its mRNA was not produced endogenously in NIH3T3 cells. Since LIF is a secretory protein, expression of LIF was also determined by measuring the amount of LIF in the culture media. Western blot analysis using anti-LIF antibody showed that the amount of LIF secreted from NIH3T3 cells increased with time, peaked on day 4, and was maintained at a high level thereafter (Fig. 5C). A band appeared at approximately 45 kDa, which was higher than the 22 kDa expected for non-glycosylated LIF produced in *E. coli*. This phenomenon is most likely due to glycosylation of LIF by posttranslational modification. The biological activity of LIF was assessed by its capability to induce differentiation

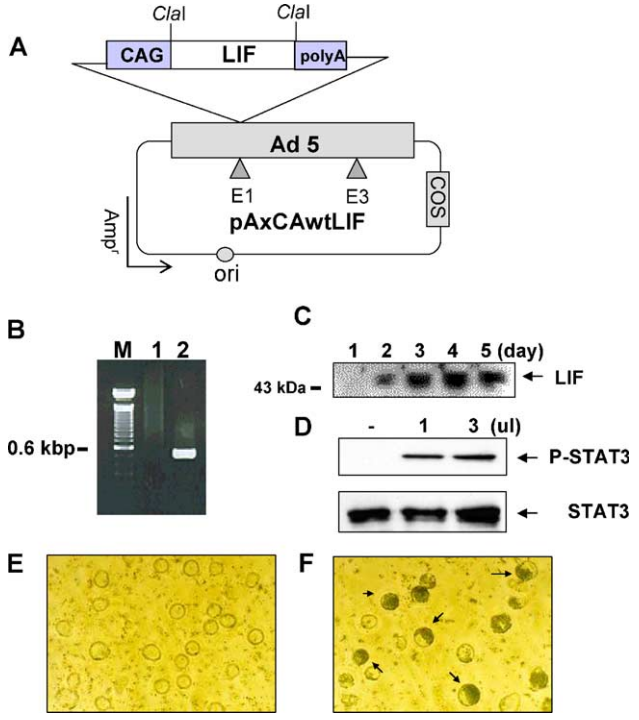

Fig. 5. The production of a recombinant adenovirus LIF and the measurement of its biological activity. (A) Recombinant adenovirus encoding LIF (Ad-LIF) was constructed using cosmid pAxCAwt, propagated in 293 cells, and infected into NIH3T3 cells to express LIF. (B–C) The existence of LIF mRNA and protein was detected by RT-PCR (B) and Western blot analysis (C), respectively. In (B), 1 and 2 indicate the cells infected without or with Ad-LIF, respectively, and M shows a 100-bp DNA marker (Gibco BRL). (D) M1 cells were treated with the culture media of NIH3T3 cells infected with Ad-LIF for 10 min. Total protein fraction was prepared as described in Materials and methods, and STAT3 phosphorylation was examined by Western blot analysis using anti-phosphospecific STAT3 antibody. (E–F) Biological activity of glycosylated-LIF was determined by the phagocytotic activity of M1 cells following LIF treatment. Cells were treated with the culture medium of Ad-LacZ- (E) and Ad-LIF- (F) infected NIH3T3 cells. Bright-field images were seen at ×200. Arrows indicate the differentiated M1 cells, which accumulated microbeads in the cytosol of cells.

of M1 myeloid cells and activate STAT3. Differentiated M1 myeloid cells engulf microbeads and accumulate them in the cytosol [32]. As seen in Fig. 5E and F, many microbeads were seen in the cytosol of the cells following treatment for 12 h with LIF produced from Ad-LIF (Fig. 5F). In addition, LIF could induce phosphorylation of STAT3 (Fig. 5D). Thus, these results indicated that the LIF gene delivered into NIH3T3 cells via adenovirus was transcribed, and LIF protein was secreted into the culture medium in a biologically active form.

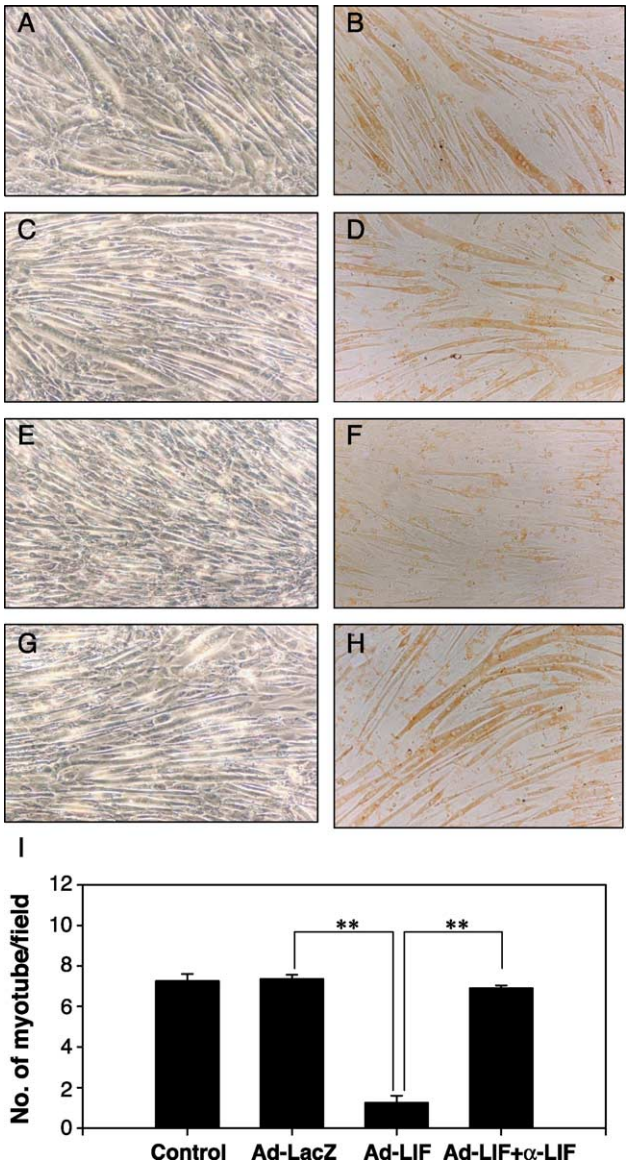

Fig. 6. Glycosylated LIF also inhibits differentiation of the C2C12 myoblast. C2C12 cells were induced to differentiate by switching the GM to DM and treated for 3 days with the culture media of NIH3T3 cells (1.5 μl/ml) which is infected with Ad-LacZ (C, D), Ad-LIF (E, F), or not treated (A, B). (G, H) indicate the culture media (1.5 μl/ml) of NIH3T3 cells infected with Ad-LIF preincubated with anti-LIF antibody (500 μg/ml) overnight. The cells were immunostained with anti-MHC antibody (MF20) and the corresponding secondary antibody. (I) shows the quantitation of the MHC immunopositive myotubes per field (×100). The results are shown as the mean±S.D. of triplicate experiments. (**P<0.01).

3.5. Glycosylated LIF and non-glycosylated LIF exhibits similar effects on myogenesis

Although the biological activity of glycosylated LIF has been reported to be similar to that of non-glycosylated one, LIF is synthesized and shows its activity as a highly glycosylated form in vivo [33]. Thus, we examined if the LIF-mediated inhibitory effect on myoblast differentiation was also shown by glycosylated LIF. To do this, C2C12 cells grown in the GM were switched to DM and treated with glycosylated LIF. We found that the ED_{50} was approximately 0.5 μl/ml of medium and the cells were routinely treated with 1.5 μl/ml of medium. Three days after treatment, the cells were immunostained with anti-MHC antibody as described in Materials and methods. The MHC immunopositive cells which are considered to be differentiated myotubes were barely seen in the dishes treated with LIF (Fig. 6E and F) while many cells were observed in the untreated control dishes (Fig. 6A and B), indicating that glycosylated LIF also inhibited differentiation of muscle cells. Quantitation of the MHC immunopositive myotubes is shown in Fig. 6I. This LIF-induced inhibitory effect on myogenesis was apparently LIF-specific since C2C12 cells treated with the culture media of NIH3T3 cells infected with Ad-LacZ were differentiated properly in DM (Fig. 6C and D). To identify further if this LIF-induced response was mediated directly by LIF or indirectly by unidentified cytokines or growth factors released from NIH3T3 cells in response to Ad-LIF infection in an autocrine manner, we tested the ability of anti-LIF neutralizing antibody to block the LIF-induced response. Preincubation of glycosylated LIF with anti-LIF antibody (500 μg/ml) at 4 °C overnight reversed completely the LIF-induced inhibition of myogenesis (Fig. 6G and H).

4. Discussion

In the present study, we demonstrated that LIF inhibited differentiation of C2C12 muscle cells. When myoblasts grown in the GM were switched to DM, a myogenic program was activated, and myogenesis occurred as revealed by an increase of MHC expression, MCK transcriptional activity and myotube formation. However, treatment of myoblasts with LIF at the onset of differentiation clearly inhibited myogenesis (Figs. 1 and 6). In addition, glycosylated LIF showed a similar biological activity to non-glycosylated LIF, suggesting that the LIF-induced inhibitory effect on muscle differentiation is independent of glycosylation. This inhibition was apparently LIF-specific because a neutralizing antibody against LIF indeed abolished this effect (Fig. 6G and H). Furthermore, although the functional role of the ERK signaling pathway in myogenesis has been controversial [23–28], our data support the conclusion that activation of the ERK signaling pathway is likely to induce inhibition of muscle differentiation.

Growth factors such as basic fibroblast growth factor (bFGF), IGF-I, and transforming growth factor β (TGF-β) as well as LIF have been known to regulate proliferation and differentiation of myoblasts [15,16,34]. IGFs not only promote proliferation of myoblasts, but also stimulate differentiation of them. In contrast, bFGF and TGF-β stimulate proliferation of myoblasts to a differential extent, but inhibit their differentiation and fusion. As shown in the present study, and from the previous reports, the biological action of LIF in muscle cells seems to be similar to bFGF and TGF-β in that LIF stimulates proliferation of myoblasts [15–17] while it inhibits differentiation of them.

Although the intracellular signaling pathway underlying myogenesis is not yet fully understood, recent studies have shown that inhibition of muscle differentiation by bFGF is mediated by the ERK pathway [25,26]. In addition, MEK1, an upstream component of ERK, was suggested as a key molecule involved in such repression. Activated MEK1 binds to the nuclear MyoD transcriptional complex and thereby inhibits the MyoD induced transactivation, resulting in suppression of myogenesis [24]. In this regard, we also observed that LIF activates ERK in as little as 10 min (Fig. 2A) and that a specific MEK inhibitor U0126 completely blocked the LIF-induced ERK phosphorylation (at 10 μM, Fig. 2B) and reversed inhibition of myogenesis (at 3 μM, Fig. 2C and D). In addition, the repressed transcriptional activity of MCK by LIF was reversed in cells transiently transfected with DN-MEK1 (Fig. 2F). Thus, our data strongly support the conclusion that inhibition of muscle differentiation results in part from the activation of the ERK signaling pathway.

A very recent publication demonstrated that STAT3 inhibits myogenic differentiation though direct interaction with MyoD [29]. In our study, however, the repression in the MCK promoter activity induced by LIF was not recovered by pretreatment of a Jak kinase inhibitor, AG490. Moreover, the basal MCK activity in cells not treated with LIF was also repressed in the presence of AG490 (Fig. 3C). Additionally, the repressed MCK promoter activity by LIF was not reversed in cells transiently transfected with DN-STAT3, or PIAS3, a STAT3 inhibitor (Fig. 3D). Taken together, these results suggest that STAT3 activation is not involved in LIF-mediated suppression of myogenesis and rather it could be required in myogenesis. This discrepancy between the two studies is not well understood at the moment. However, in the previous study, activation of STAT3 in the cells was introduced by transfecting a chimeric receptor composed of the extracellular domain of granulocyte-colony stimulating factor receptor and the cytoplasmic domain of gp130 or STAT3-C (constitutively active STAT3). In contrast, in our study, STAT3 activation was induced through activation of the endogenous receptor complexes by LIF treatment. Thus, while massive STAT3 activation by transfection of a chimeric receptor or STAT3-C could lead to the repression of differentiation abnormally, LIF-mediated endogenous STAT3 activation may not be strong enough to induce such effect.

Our result conflicts with another previous report showing that LIF had no significant effect on differentiation of muscle satellite cells [18]. However, we confirmed that LIF clearly inhibited myoblast differentiation. Interestingly, this repressive action by LIF was observed only in the cells induced to differentiate for 0–12 h in the DM, but not seen in the cells grown in the GM or induced for 24 h in the DM. Thus, based on our observation, if differentiation is induced 5 days after LIF treatment as done in the previous study [18], we may not see the LIF-induced repressive effect on myoblast differentiation. Our results are consistent with another study that there is a critical time window (16–20 h) during which bFGF-induced ERK activity is able to repress myogenic gene expression and differentiation [25].

LIF is a glycoprotein containing several N-linked glycosylation sites and is extracellularly secreted [33]. Most of the previous studies concerning LIF effects on muscle have used bacterially expressed non-glycosylated LIF. Although it is uncertain if the activity of the non-glycosylated form is lower than that of the glycosylated form, no difference between both forms has been reported in various biological tests such as activation of STAT3, growth inhibition of leukemia cells and differentiation of them to macrophages, and cholinergic neuronal differentiation [35,36]. However, it was reported that the Man-6-P moiety of glycosylated LIF binds to Man-6-P/IGF-II receptor, which results in internalization and degradation of the cytokine, suggesting that glycosylation plays a crucial role in the metabolism of LIF [37]. As demonstrated in the present study, both glycosylated and non-glycosylated LIF had a similar effect on myogenesis. Nevertheless, use of the glycosylated form, especially in animal studies, could eliminate any possible discrepancy in biological responses elicited by the glycosylated or non-glycosylated form of LIF.

Regeneration of adult skeletal muscle stems from muscle precursor cells, which are normally present as quiescent satellite cells between the basement membrane and the plasma membrane of myofibres [38]. These cells become activated following injury, exercise, or denervation as well as in diseased states. The activated satellite cells, which are essentially myoblasts, then proliferate, differentiate and eventually fuse to form myotubes [39]. Although LIF has been implicated in muscle regeneration and thus considered as an attractive therapeutic agent for injured muscles [11,12], other reports have suggested no beneficial effect of LIF on muscle regeneration or myotube formation [13,14]. While previous publications showed that LIF induces proliferation of myoblasts or muscle satellite cells in culture [15–17], our data demonstrated that it inhibits muscle differentiation (Figs. 1 and 6). Thus, in light of muscle regeneration in vivo, these contrasting effects of LIF on muscle cells in vitro suggest its stage-dependent role in the course of regeneration of damaged muscles. We

conclude that LIF is beneficial only during muscle satellite cell proliferation, but is unfavourable thereafter. Future studies are necessary to elucidate its precise role in muscle regeneration in vivo.

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